Universitätsspital Zürich
Klinik für Endokrinologie, Diabetologie und klinische Ernährung
Klinikdirektor: Prof. Dr. med. G.A. Spinas
Bereich IV Leiter: Prof. Dr. med. M. Fried
Departement für Innere Medizin

Arbeit unter der Leitung von PD. Dr. med. P. Wiesli

Decreased macrovascular complications in diabetic patients treated with aspirin due to genetic variants influencing aspirin metabolism

INAUGURAL-DISSERTATION
zur Erlangung der Doktorwürde der Medizinischen Fakultät
der Universität Zürich

vorlgelegt von
Marianne Schmid
von Glattfelden ZH

Genehmigt auf Antrag von Prof. Dr. med. G.A. Spinas
Zürich 2009

Inhaltsverzeichnis:

Seite:

1. Abstract

Background: There is increasing evidence of aspirin resistance for the prevention of cardiovascular events in a substantial proportion of diabetes mellitus. Genetic variants influencing aspirin metabolism may contribute to the failure of aspirin therapy in patients with diabetes. Aspirin is metabolized by uridine diphosphate glucuronosyltransferase isoenzyme 1A6 (UGT1A6). A missense mutation, T181A of the UGTA6, has been associated with 30-50% lower enzyme activity as compared to the wild-type.

Methods: In this cross-sectional pilot study, macrovascular complications were assessed as a function of the UGT1A6 polymorphism in 74 diabetic patients on aspirin therapy.

Results: Of the 74 diabetic patients treated with 100mg aspirin daily, 48 (65%) carried the UGT1A6 polymorphism (37 with allele TA; 11 with TT allele) and 26 (35%) the wild-type (AA). The prevalence of macrovascular complications was 35% in carriers of UGT1A6 polymorphism as compared to 62% in wild-type carriers ($p=0.031$). In a multiple regression analysis considering possible confounding variables, the prevalence of macrovascular complications remained significantly associated with the UGT1A6 polymorphism.

Conclusions: UGT1A6 polymorphism may be associated with decreased macrovascular complications in diabetic patients treated with aspirin.

2. Introduction

In patients with diabetes, prescription of low-dose aspirin for the prevention of cardiovascular events is well established (1, 2, 3), although this measure is still debated. A recent meta-analysis did not show a significant preventive effect or aspirin in diabetic patients (4) and, in primary prevention, low-dose aspirin was less effective in diabetic patients compared to patients with other risk factors.
Aspirin metabolism may contribute to the efficacy of aspirin therapy. The elimination rate of aspirin and its metabolic salicylic acid is likely a major factor determining drug efficacy among aspirin users. Salicylic acid is glucuronidated by the uridine diphosphate glucuronosystransferase isoenzyme 1A6 (UGT1A6) and glucuronidation activity may be a critical determinant in aspirin elimination (6). The common missense mutation, T181A of the UGT1A6, has been associated with 30-50% lower enzyme activity compared with the wild-type (7) in vitro. In a clinical context among aspirin users, carriers of the allelic UGT1A6 showed a decreased risk of colorectal adenoma in patients with slower glucuronidation of aspirin (8, 9).

In this cross-sectional pilot study, we assessed macrovascular complications as a function of the UGT1A6 polymorphism in diabetic patients on aspirin therapy.

3. Methods

Patients and Methods

Patients with diabetes attending the University Hospital of Zurich as out-patients, were invited to participate. Inclusion criteria were diabetes mellitus and treatment with aspirin 100mg daily. Exclusion criteria for the study were acute or chronic inflammatory disease, cancer, immunosuppression, and treatment with glucocorticoids or glitazones. The study population was the same as in a previous study (10), with the exception of 2 patients who were excluded because they were treated with 300mg aspirin daily. The study protocol was approved by the Ethics Committee of the University Hospital of Zurich, and all patients gave written informed consent.

Assessment of clinical parameters and laboratory work were carried out independently by different and blinded study physicians. The occurrence of macrovascular complications under aspirin therapy were assessed retrospectively from medical records. Coronary artery disease was defined as history or either myocardial infarction, coronary angioplasty or bypass surgery; cerebrovascular disease as history of apoplexy or carotid artery surgery; peripheral artery disease as history of angioplasty or bypass surgery of peripheral arteries. Retinopathy, neuropathy, and nephropathy were assessed from the medical records as established diagnoses. Genomic DNA was isolated from peripheral blood mononuclear cells. GHR exon 3 and TNF-α-308 promoter genotyping were carried out on a LightCycler (Roche Molecular Biochemicals, Rotkreuz, Switzerland) using ToolSets containing specific primers and fluorescent probes (Genes-4U AG, Zurich) according to the manufacturer's instructions. Serum concentrations of hs-C-reactive protein was determined on an Immulite 2000 Immunology Analyzer using commercial assays from DPC (Los Angeles, CA, USA). TNF- α, Interleukin 1-β and interleukin 6 were determined by Luminex technology using commercially available multianalyte profiling kits (R&D Systems) and solid-phase enzyme amplified sensitivity immunoassay (EASIA), respectively.

Statistics

Statistical analysis was performed using Statistica version 6. Variables were compared using either Student's t-test, Mann-Whitney U-test, or X^2-test. Multiple regression analysis was performed on the number of patients with macrovascular complications. Variables with uncertain influence ($p>0.10$) were excluded during analysis using a stepwise backward elimination procedure. The threshold of significance was defined at $\alpha=0.05$.

4. Results

Clinical and biochemical characteristics of 74 included patients with diabetes and treatment with aspirin 100mg daily are given in Table 1. 48 (65%) of the 74 patients carried the UGT1A6 polymorphism (37 heterozygous, allele TA; 11 homozygous, TT allele) and 26 (35%) the wild-type (AA). This corresponds to a mutated UGT1A6 allele frequency of 40%. Carriers of the UGT1A6 wild-type were more often male than those carrying the UGT1A6 polymorphism. With regard to other clinical characteristics, cardiovascular risk factors and medical therapy (Table 1 and 2), carriers of the UGT1A6 polymorphism and wild-type were well balanced. Whereas hs-C-reactive protein, TNF-α and interleukin 6 were comparable between both groups, interleukin 1-β serum values were lower in patients carrying the UGT1A6 polymorphism than in wild-type carriers.

Table 3 shows the frequency of macrovascular complications depending on the UGT1A6 polymorphism. The prevalence of macrovascular complications before aspirin therapy was initiated (data not shown) was comparable between both groups. Under aspirin therapy, the incidence of macrovascular complications was 35% in carriers of the UGT1A6 polymorphism compared to 62% in carriers of the wild type (p=0.031). Accordingly, the number of macrovascular complications per patient was significantly lower in carriers of the UGT1A6 polymorphism compared to carriers of the wild-type (p=0.039).

We previously described a significant association of TNF-α G308A polymorphism on macrovascular complications in this study population (10). Therefore TNF-α G308A polymorphism was added into the stepwise multiple regression analysis - in addition to the UGT1A6 polymorphism, cardiovascular risk factors, demographic and laboratory data - on the number of patients with macrovascular complications (Table 4). Both polymorphisms remained significantly associated (TNF-α G 308A polymorphism, p=0,02; UGT1A6 polymorphism, p=0.04 with the frequency of macrovascular complications, whereas there was a trend for TNF-α (p=0.08). Male gender and Interleukin 1-β, both significant in the univariate analyses (see Table 1),

were no more significantly associated with the number of patients with macrovascular complications in the multiple regression analysis.

Figure 1 shows the proportion of patients with macrovascular complications under aspirin therapy according the UGT1A6 T181 A and TNF-α G308A polymorphism. Only 19% (3 of 16 patients) of patients carrying both mutations had macrovascular complications, whereas 67% (16 of 24 patients) of the wild-type carriers (p=0.003) experienced macrovascular complications. 41% (14 of 34 patients) of the patients with either UGT1A6 T181 or TNF-α G 308A polymorphism experienced macrovascular complications, i.e. significantly less patients compared to the wild-type carriers (p=0.049)

Table 1. Characteristics of 74 patients with diabetes mellitus.

	UGT1A6 T181A polymorphism		
	mutation* (n=48)	wild type (n=26)	p-value
Age, y	65 ± 10	65 ± 9	0.88
Male gender, no. (%)	28 (58)	22 (84)	0.021
Body-mass index, kg/m2	28.5 ± 5.4	27.6 ± 4.0	0.42
Diabetes duration, y	16.5 ± 12.5	15.5 ± 14.0	0.74
Diabetes type 1, no. (%)	9 (19)	6 (23)	0.66
Diabetes type 2 with insulin therapy, no. (%)	22 (46)	10 (38)	0.54
Diabetes type 2 without insulin therapy, no. (%)	17 (35)	10 (38)	0.80
Hemoglobin A1c, %	7.5 ± 1.1	7.2 ± 0.8	0.12
Retinopathy, no. (%)	21 (44)	8 (31)	0.22
Neuropathy, no. (%)	36 (75)	20 (77)	0.85
Nephropathy, no. (%)	23 (48)	14 (54)	0.63
Duration of aspirin therapy, y	4.75 ± 4.6	5.5 ± 5.8	0.56
Smoking (former or persistent), no.	26 (54)	17 (65)	0.35
Arterial Hypertension, no. (%)	41 (85)	22 (85)	0.93
Total cholesterol (mmol/L)	4.96 ± 0.86	4.73 ± 0.78	0.28
LDL-cholesterol (mmol/L)	2.76 ± 0.79	2.74 ± 0.67	0.93
HDL-cholesterol (mmol/L)	1.43 ± 0.43	1.32 ± 0.49	0.33
Triacylglycerols (mmol/L)	1.67 ± 0.94	1.45 ± 0.58	0.30
hs-C-reactive protein, mg/L	2.7 (0.3-50.1)	1.8 (0.5-56.6)	0.36
TNF-α, ng/L	6.4 (1.4-12.2)	6.4 (4.0-31)	0.97
Interleukin 1-β, ng/L	1.0 (0.2-2.5)	1.4 (0.02-3.4)	0.010
Interleukin 6, ng/L	4.9 (1.3-15.3)	4.3 (1.9-10.5)	0.23

Values are mean ± SD, numbers of patients (%) or medians with range; p-values were calculated by Student's t-test, χ^2 test or Mann Whitney U Test. * 37 heterozygotes (T181/A181), 11 homozygotes (A181/A181).

Table 2. Actual therapy of 74 patients with diabetes under aspirin therapy.

	UGT1A6 T181A polymorphism		
	mutation (n=48)	wild type (n=26)	p-value
β Blocker, no. (%)	28 (58)	13 (50)	0.49
ACE Inhibitors, no. (%)	27 (56)	16 (62)	0.66
AT II-Receptor Inhibitors, no. (%)	9 (19)	3 (12)	0.42
Calcium antagonist, no. (%)	8 (17)	7 (27)	0.29
Diuretics, no. (%)	19 (40)	10 (38)	0.92
Statins, no. (%)	31 (65)	20 (77)	0.27
Nitrates, no. (%)	8 (17)	1 (4)	0.11

Values are numbers of patients (%); p-values were calculated by χ^2 test.

Table 3. Macrovascular complications of 74 diabetic patients under low dose aspirin therapy as a function of polymorphism UGT1A6 T181A polymorphism.

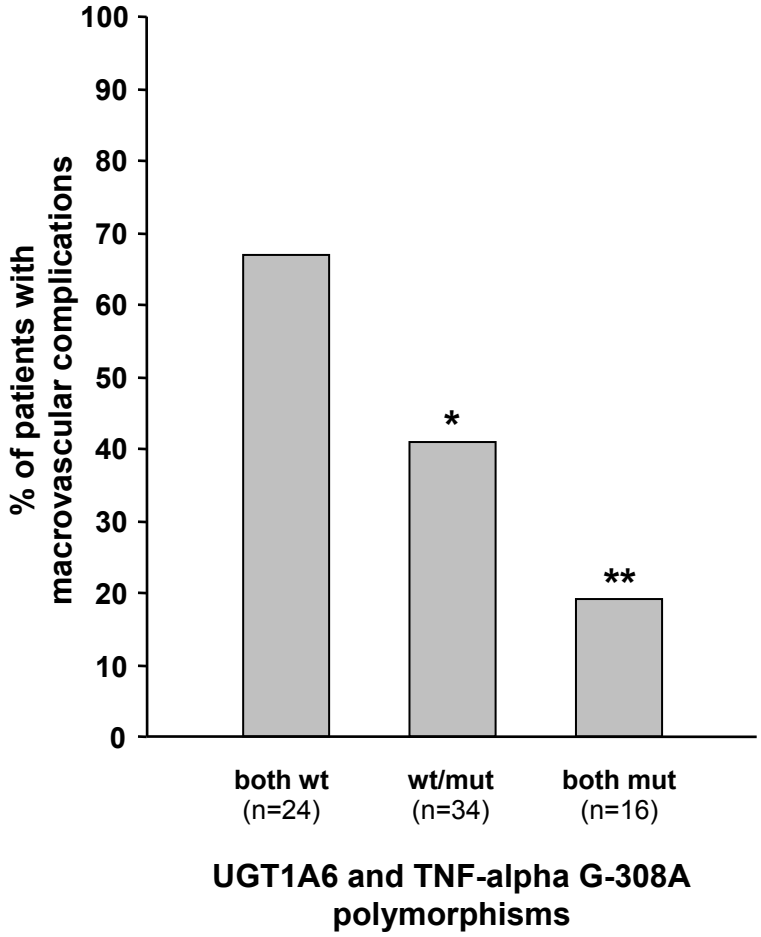
		UGT1A6 T181A polymorphism		
		mutation (n=48)	wild type (n=26)	p-value
Macrovascular complications of atherosclerosis under low dose aspririn therapy, no. of patients (%)				
- All patients	**(n=74)**	17 (35)	16 (62)	**0.031**
- Male patients	**(n=50)**	9 (32)	13 (59)	0.06
- Female patients	**(n=24)**	8 (40)	3 (75)	0.20
- Coronary (myocardial infarction, angioplasty or bypass surgery		12 (25)	9 (35)	
- Peripheral arteries (percutanous angioplasty)		8 (17)	7 (27)	
- Cerebrovascular (apoplexy or carotid endarterectomy)		2 (4)	4 (15)	
Number of complications, no. of patients (%):				**0.039**
0		26 (75)	10 (38)	
1		12 (25)	9 (35)	
≥ 2		5 (10)	7 (27)	

P-values were calculated by χ^2 test and Mann-Whitey U Test (Number of complications).

Table 4. Stepwise, multiple regression analysis of cardiovascular risk factors, demographic and laboratory data on the number of patients with atherosclerotic manifestations under aspirin therapy.

	Number of patients with atherosclerotic manifestations	
	β	**p-value**
Variables remaining in the model		
TNF-α promoter G308A polymorphism	-0.29	**0.020**
UGT1A6 T181A polymorphism	-0.24	**0.040**
Log TNF-α, ng/L	0.20	**0.08**
Variables excluded from the model		
Statine treatment	0.17	0.14
Cholesterol/HDL-Ratio	0.16	0.16
Body-mass index	-0.16	0.19
Smoking (former and current)	0.12	0.28
Type of diabetes	0.16	0.29
Diabetes duration	0.12	0.30
Log Hemoglobin A1c	-0.12	0.35
Log Interleukin 6, ng/L	-0.13	0.36
Log hs-C-reactive protein, mg/L	0.11	0.39
Log Interleukin 1-β, ng/L	-0.13	0.41
Male gender	-0.09	0.44
Insulin therapy	-0.08	0.54
Log Trialcylglycerols	-0.05	0.78
Arterial Hypertension	0.01	0.91
Age	-0.01	0.94

Figure Percent of diabetic patients (n=74) with macrovascular complications under aspirin therapy according the UGT1A6 T181A and TNF-alpha G-308A polymorphisms. Wt/mut stands for patient with either UGT1A6 T181 or TNF-alpha G-308A polymorphisms. * denotes $p<0.05$, ** $p<0.01$.



5. Discussion

In the present study population of diabetic patients treated with 100mg aspirin, the UGT1A6 polymorphism was significantly associated with the frequency of macrovascular complications. The incidence of macrovascular complications was lower in carriers of the UGT1A6 allele (TA+AA) than in homozygous carriers of wild-type alleles (TT). 62% of patients with the UGT1A6 wild-type versus 35% with mutated UGT1A6 polymorphism experienced macrovasular complications despite aspirin therapy. Aspirin and its metabolism salicylic acid are glucuronidated by UGT1A6 (6). Lower enzyme activity in carriers of the UGT1A6 polymorphism may lead to diminished drug elimination and therefore enhanced drug efficacy among aspirin users. UGT1A6 polymorphism has been shown to be associated with 30-50% lower enzyme activity compared with the wild-type (7).

Inhibition of cyclooxygenase-1 (COX-1) and therefore thromboxane (TXA2)-dependent platelet activation has been claimed to be the major mechanism of aspirin (ASA) in the prevention of cardiovascular events. Pronounced (ASA-) drug efficiency in patients with UGT1A6 polymorphism is however unlikely to be explained by the COX-1 pathway because even small amount of ASA can cause complete COX-1-blockade with no further benefit with higher ASA dosages (12). Thus, COX-1 independent ASA effects may be responsible for the decreased incidence of macrovascular complications in carriers of the UGT1A6 polymorphism. COX-2 is mainly activated by inflammatory processes in monocytes/macrophages or shear stress in vascular endothelial cells. Increased (locally) ASA concentrations in patients with mutated UGT1A6 would result in less pro-atherogenic TXA2 production and enhanced locally anti-inflammatory ASA effects (13). Furthermore enhanced aspirin COX-2 triggered generation of lipoxin - a recently found anti-inflammatory mediator - may result (14). Other possible aspirin sensitive mechanisms include diminished release of pro-inflammatory mediators such as tissue factors (TF) (15), soluble CD40 ligand (16, 17), reactive oxygen species (ROS) (18) or nuclear factor kB (19). We expected lower inflammatory markers in patients carrying the UGTA61 polymorphisms. However, interleukin 1- β serum levels were found to be lower in carriers of the UGT1A6 polymorphism.

We previously reported an association of TNF-α promoter G308A polymorphism and macrovascular complications in the same study population. Patients with the TNF-α promoter G308A polymorphism exhibited lower inflammatory cytokines (such as hs-C-reactive protein) than wild-type carriers. Using multiple regression analysis, both polymorphisms remained significantly associated with the incidence of macrovascular complications, i.e. carriers of both mutations had a lower incidence of macrovascular complications compared to wild-type carriers (figure).

There are several limitations to our study. First we did not measure aspirin metabolites. However, measurement of aspirin or its metabolites is not particularly informative because of rapid clearance in the gut and exerting their effects mainly in the presystemic circulation (21). In addition, we did not assess platelet function. Whether changes in these functional tests accurately reflect platelet activation and inhibition in vivo, however, is still unclear (22). Moreover, the correlation between results of different tests on aspirin responsiveness is poor (23). Another limitation of our study is a gender inbalance, i.e. we had more male patients carrying the mutated AGT1A6 alleles than wild-type carriers. Nevertheless, multiple regression analyses showed no influence of gender regarding macrovascular complications. The major limitation of our study is the small number of patients which warrants confirmation by additional studies. Finally, we cannot exclude that our observations reflect associations with other additional gene variants rather than the investigated polymorphisms. However, heritable factors may be crucial concerning individual aspirin responsiveness, as recently shown by others (24).

In conclusion we could show for the first time that UGT1A6 polymorphism may be associated with the frequency of macrovascular complications in diabetic patients.

6. References

1. Haffner SM. Coronary heart disease in patients with diabetes. N Engl J Med 2000; 342:1040-2.

2. Knopp RH, Retzlaff B, Aikawa K, Kahn SE. Management of patients with diabetic hyperlipidemia. Am J Cardiol 2003; 91(7A):24E-28E.

3. Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. Diabetes Care 2003; 26:2181-8.

4. Antithrombotic Trialists' Collaboration: Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 2002; 324:71-86.

5. Sacco M, Pellegrini F, Roncaglioni MC, Avanzini F, Tognoni G, Nicolucci A; PPP Collaborative Group. Primary prevention of cardiovascular events with low-dose aspirin and vitamin E in type 2 diabetic patients: results of the Primary Prevention Project (PPP) trial. Diabetes Care 2003; 26:3264-72.

6. Kuehl GE, Bigler J, Potter JD, Lampe JW. Glucuronidation of the aspirin metabolite salicylic acid by expressed UDP-glucuronosyltransferases and human liver microsomes. Drug Metab Dispos 2006; 34(2):199-202.

7. Ciotti M, Marrone A, Potter C, Owens IS. Genetic polymorphism in the human UGT1A6 (planar phenol) UDP-glucuronosyltransferase: pharmacological implications. Pharmacogenetics 1997; 7(6):485-95.

8. Bigler J, Whitton J, Lampe JW, Fosdick L, Bostick RM, Potter JD. CYP2C9 and UGT1A6 genotypes modulate the protective effect of aspirin on colon adenoma risk. Cancer Res 2001; 61(9):3566-9.

9. Chan AT, Tranah GJ, Giovannucci EL, Hunter DJ, Fuchs CS. Genetic variants in the UGT1A6 enzyme, aspirin use, and the risk of colorectal adenoma. J Natl Cancer Inst 2005; 97(6):457-60.

10. Krayenbuehl PA, Wiesli P, Schmid M, Schmid C, Ehses JA, Hersberger M, Vetter W, Schulthess G. TNF-alpha -308G>A polymorphism modulates cytokine serum concentrations and macrovascular complications in diabetic patients on aspirin. Exp Clin Endocrinol Diabetes 2007; 115(5):322-6.

11. Lampe JW, Bigler J, Horner NK, Potter JD. UDP-glucuronosyltransferase (UGT1A1*28 and UGT1A6*2) polymorphisms in Caucasians and Asians: relationships to serum bilirubin concentrations. Pharmacogenetics 1999; 9(3):341-9.

12. Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. J Clin Invest 1982; 69(6):1366-72.

13. Kobayashi T, Tahara Y, Matsumoto M, Iguchi M, Sano H, Murayama T, Arai H, Oida H, Yurugi-Kobayashi T, Yamashita JK, Katagiri H, Majima M, Yokode M, Kita T, Narumiya S.
Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. J Clin Invest 2004; 114(6):784-94.

14. Chiang N, Bermudez EA, Ridker PM, Hurwitz S, Serhan CN. Aspirin triggers antiinflammatory 15-epi-lipoxin A4 and inhibits thromboxane in a randomized human trial. Proc Natl Acad Sci U S A 2004; 101(42):15178-83.

15. Rao AK, Vaidyula VR, Bagga S, Jalagadugula G, Gaughan J, Wilhite DB, Comerota AJ.
Effect of antiplatelet agents clopidogrel, aspirin, and cilostazol on circulating tissue factor procoagulant activity in patients with peripheral arterial disease. Thromb Haemost 2006; 96(6):738-43.

16. Nannizzi-Alaimo L, Alves VL, Phillips DR. Inhibitory effects of glycoprotein IIb/IIIa antagonists and aspirin on the release of soluble CD40 ligand during platelet stimulation.
Circulation 2003; 107(8):1123-8.
17. Santilli F, Davi G, Consoli A, Cipollone F, Mezzetti A, Falco A, Taraborelli T, Devangelio E, Ciabattoni G, Basili S, Patrono C. Thromboxane-dependent CD40 ligand release in type 2 diabetes mellitus. J Am Coll Cardiol 2006;47(2):391-7.
18. Nascimento-Silva V, Arruda MA, Barja-Fidalgo C, Fierro IM. Aspirin-triggered lipoxin A4 blocks reactive oxygen species generation in endothelial cells: a novel antioxidative mechanism. Thromb Haemost 2007; 97(1):88-98.
19. Weber C, Erl W, Pietsch A, Weber PC. Aspirin inhibits nuclear factor-kappa B mobilization and monocyte adhesion in stimulated human endothelial cells.
Circulation 1995; 91(7):1914-7.
20. Sacco M, Pellegrini F, Roncaglioni MC, Avanzini F, Tognoni G, Nicolucci A; PPP Collaborative Group. Primary prevention of cardiovascular events with low-dose aspirin and vitamin E in type 2 diabetic patients: results of the Primary Prevention Project (PPP) trial. Diabetes Care 2003; 26:3264-72.
21. Maree AO, Fitzgerald DJ.Variable platelet response to aspirin and clopidogrel in atherothrombotic disease. Circulation 2007; 115(16):2196-207.
22. Patrono C, Rocca B. Drug insight: aspirin resistance--fact or fashion? Nat Clin Pract Cardiovasc Med 2007; 4(1):42-50.
23. Harrison P, Segal H, Blasbery K, Furtado C, Silver L, Rothwell PM. Screening for aspirin responsiveness after transient ischemic attack and stroke: comparison of 2 point-of-care platelet function tests with optical aggregometry.
Stroke 2005; 36(5):1001-5.

24. Faraday N, Yanek LR, Mathias R, Herrera-Galeano JE, Vaidya D, Moy TF, Fallin MD, Wilson AF, Bray PF, Becker LC, Becker DM. Heritability of platelet responsiveness to aspirin in activation pathways directly and indirectly related to cyclooxygenase-1. Circulation 2007; 115(19):2490-6.

7. Verdankungen

Ich möchte allen, die mir die Arbeit an der Dissertation erleichterten und ermöglichten, ganz herzlich Danken.

- Peter Wiesli, für seine Betreuung und seine Geduld.
- Dem Endokrinologie-Team des Universitätsspitals Zürich, besonders Pierre-Alexandre Krayenbühl für seine tatkräftige Unterstützung.
- Meiner Familie für die moralische Unterstützung.

8. Curriculum vitae

Persönliche Daten

Name	Marianne SCHMID
Geburtsdatum	27.02.1977
Geburtsort	Bülach (ZH)
Bürgerort	Glattfelden (ZH)
Zivilstand	ledig

Ausbildung

1984-1990	Primarschule Glattfelden
1990-1992	Sekundarschule Glattfelden
1992-1997	Gymnasium KZU Bülach
1997	Eidgenössische Matura Typus B
1997-2004	Studium der Humanmedizin an der Universität Zürich
2004	Eidgenössisches Staatsexamen

Weiterbildung

05/05-09/06	Assistenzärztin Chirurgie Spital Zimmerberg, Horgen
Seit 1.11.2006	Assistenzärztin Anästhesiologie Kantonsspital Aarau